Cathepsin L induces proangiogenic changes in human omental microvascular endothelial cells via activation of the ERK1/2 pathway

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Abstract

Background: Metastasis still remains the major cause of therapeutic failure, poor prognosis and high mortality in epithelial ovarian cancer (EOC) patients. Previously, we showed that EOC cells secrete a range of factors with potential pro-angiogenic activity, in disease-relevant human microvascular omental endothelial cells (HOMECs), including the lysosomal protease cathepsin L (CathL). Thus, the aim of this study was to examine potential pro-proliferative and pro-migratory effects of CathL in HOMECs and the activated signalling pathways, and whether these proangiogenic responses are dependent on CathL-catalytic activity.

Methods: HOMECs proliferation was investigated using WST-1, BrdU and CyQUANT assays. Cell migration was examined using a Cultrex Cell 96 transwell migration assay. A range of pHs were assayed to assess enzyme activity in the presence of CathL-specific fluorogenic substrate FY-CHO. Activation of cell signalling pathways was tested using commercially available phosphokinase array and intact cell-based ELISAs.

Results: We show for the first time that CathL has a potent pro-proliferative and pro-migratory effect on HOMECs. For instance, CathL significantly increases HOMEC proliferation (134.8±14.7% vs control 100%) and migration (146.6±17.3% vs control 100%). Our data strongly suggests that these proangiogenic effects of CathL are mediated via a non-proteolytic manner. Finally, we show that CathL-induced activation of the ERK1/2 pathway is involved in inducing these cellular effects in HOMECs.

Conclusion: These data suggest that CathL acts as an extracellular ligand and plays an important pro-angiogenic, and thus pro-metastatic, role during EOC metastasis to the omentum, by activating the omental microvasculature, and thus can potentially be targeted therapeutically in the future.

Keywords: cathepsin L, non-proteolytic, proliferation, migration, angiogenesis

1. Introduction

Epithelial ovarian cancer (EOC), is the seventh most common cancer in women, and has a poor prognosis due to a lack of defined symptoms and therefore, early detection. Approximately 200,000 women suffer from the disease worldwide, with an estimated 125,000 deaths annually. Pathologically, ovarian tumours frequently spread initially to the omentum, facilitating further spread and leading to advanced disease with widespread metastasis at diagnosis. This presents a considerable therapeutic challenge, and as a result, overall survival ranges from 30 to 50%.

The predominant mechanism of EOC metastasis to the omentum is via the transcoelomic route within the peritoneum [1]. Ovarian tumour cells attach to the mesothelium of the omentum, invade the local tissue and then initiate angiogenesis to sustain secondary tumour growth. Tumour-induced angiogenesis, i.e. growth of new blood vessels from existing ones, requires a complex interplay between tumour and resident cells, with secretion of growth factors and chemokines that ultimately leads to activation of a pro-angiogenic phenotype in the host omental microvascular endothelial cells (ECs) and subsequently neovascularisation [2-6].

Conventionally, vascular endothelial growth factor A (VEGFA) has been known to be a major pro-angiogenic target in anti-angiogenic therapies in cancer [7, 8]. However, our previous studies have indicated that alternative EOC-secreted pro-angiogenic factors are involved in inducing angiogenic changes in the omental ECs during metastasis of ovarian cancer [9]. These data are supported by the observation that anti-VEGFA therapy (bevacizumab) has shown limited efficacy in patients with ovarian cancer [10], highlighting the need for a clearer understanding of the pro-angiogenic pathways involved.

One of the alternative proangiogenic factors we identified is cathepsin L (CathL), a cysteine endopeptidase, that physiologically plays an important role in degrading endocytosed proteins as well as intracellular proteins [11, 12]. Although CathL resides in lysosomes in its active form it has also been shown to be secreted from cancer cells by both ourselves (from EOC cells), as mentioned above [9] and others e.g. from a murine fibrosarcoma cell line KHT-LP1 [13], although the mechanism is unclear.

A possible role for secreted CathL in ovarian cancer development and metastasis has been reported in studies showing an increased level of secreted CathL in the sera of malignant EOC patients compared with patients with benign tumours and healthy controls [14]. Additionally, it has been shown to be involved in the invasion and metastasis of EOC through proteolytic degradation of the ECM, and hence was suggested to be a marker of advanced staged ovarian cancer [14]. This was supported by our previous work demonstrating that not only is CathL expressed in metastatic EOC cells in the omentum, but also that the endothelium of vessels within omentum hosting metastatic ovarian high-grade serous carcinoma expressed significantly increased CathL *in vivo* compared with omentum from control patients with benign ovarian cystadenoma [15].

Despite the emerging role for CathL in ovarian tumour development and angiogenesis, the exact involvement of extracellular CathL and the downstream cellular signalling pathways activated by the protein are still poorly understood and remain to be examined. The therapeutic challenge posed by EOC requires a fuller understanding of the process involved in secondary tumour formation within the omentum, in order to facilitate development of treatment strategies. We have previously published a technique for isolating disease relevant HOMECs [16] and here we use this cell model to (a) investigate whether CathL exerts its proliferative and/or migratory effects through a proteolytic or non-proteolytic mechanism and (b) activation of intracellular signalling cascades upstream of these functional responses to CathL. We report for the first time that CathL significantly increases HOMEC proliferation and migration via proteolytic-independent mechanisms. Additionally, we also demonstrate activation of intracellular kinases ERK1/2 and AKT(S473) as part of the signalling cascade in CathL-induced proliferation and migration in HOMECs.

2. Method

2.1. Primary cell culture

Non-malignant omental tissue samples were collected from patients undergoing surgery at the Royal Devon and Exeter NHS Foundation Trust (Exeter, United

Kingdom) with ethical approval and informed written consent. HOMECs were isolated, characterised and cultured as primary cells as previously described [16, 17]. Briefly, HOMECs were cultured in endothelial cell (EC) growth media (MV2, PromoCell, Heidelberg, Germany) supplemented with supplied growth factors, 5% (v/v) foetal calf serum (FCS) and 0.1% (v/v) gentamycin (Sigma, Poole, UK). Cells were maintained at 37°C in a humidified atmosphere supplemented with 5 % CO_2 [17].

2.2. Cell proliferation assay

2.2.1 WST-1 assay

Investigation of HOMEC proliferation was as previously described [17]. Briefly, cells were seeded at a density of $1x10^4$ cells per well in 2% (w/v) gelatin (Sigma, Poole, UK) coated 96-well plates (Greiner Bio One, Stonehouse, UK) and treated overnight in growth factor-deprived media containing 2% (v/v) FCS. After 24 hours, treatments (recombinant CathL (from human liver) 50ng/ml, $VEGF_{165}$ (20ng/ml as positive control ± inhibitors) were added at the given concentrations (Table 1) and incubated for 72 hours. Subsequently, WST-1 reagent (Roche, Welwyn Garden City, UK) was added to the assay medium and absorbance was measured at 450 nm against the blank in a PHERAstar BMG plate-reader [17].

2.2.2 BrdU assay

Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 20,000cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without CathL (50 ng/ml) and incubated for 48 hours. A commercially available BrdU reagent (Merck Chemicals Ltd., Nottingham, UK) was added to the wells for the last 24 hour incubation and cellular proliferation was assessed (according to the manufacturer's instructions) based on fluorescence intensity using a SpectraMax plate-reader (Molecular Devices, Berkshire, UK) at Ex/Em of 450/550 nm.

2.2.3 CyQUANT assay

This procedure was performed according the manufacturers instruction. Briefly, after 72 hour treatment with CathL (50 ng/ml), media was removed from each well, followed by addition of the dye binding solution (1X HBSS buffer) containing CyQUANT NF dye reagent (Fisher Scientific, Loughborough, UK). After 2 hour incubation, the plates were

read at Ex/Em: 485/530 using a FLUOstar BMG plate-reader (BMG Labtech Ltd, Bucks, UK) and cell proliferation was assessed based on the fluorescence intensity against the background containing HBSS buffer.

2.3. Examination of the dependence of exogenous CathL activity on pH

The following experiments were carried out as previously described [17].

2.3.1. Measurement of pH of cell culture media during cell culture

HOMECs were seeded at a density of $3x10^5$ cells per well in 6 well plates, based on preliminary experiments. After overnight incubation in growth factor depleted media as above, fresh media supplemented ± CathL (50ng/ml) was added. The concentration of CathL was derived from cell proliferation experiments as discussed later. Culture media was collected, and pH was measured after 24, 48 and 72 hours using an ABL80 FLEX blood-gas analyser (Radiometer, Crawley, UK). pH of medium-only was also measured at the beginning of incubation period.

2.3.2 Measurement of enzymatic activity of CathL at different pHs

CathL proteolytic activity was measured using a CathL-specific fluorogenic substrate Z-Val-Val-Arg-AMC, (ZVA, 5µmol/l, Enzo Life Sciences, Exeter, UK), in the presence or absence of the CathL inhibitor FY-CHO (10 µmol/l) in 96-well plates (Greiner Bio One, Stonehouse, UK). Prepared buffer solutions at specific pHs containing ZVA ± FY-CHO were added to the wells (100 µl). Subsequently, 20 µl of 300 ng/ml CathL was added as required to make up the final volume of 120 µl (50 ng/ml CathL). Control wells contained ZVA or ZVA plus FY-CHO, and 20 µl of corresponding pH buffer solution. The plate was shaken for 60 seconds in a plate-reader immediately prior to fluorescence reading at Ex/Em: 365/440. The experiment was performed away from direct light exposure. The pH buffer solutions were prepared by mixing citric acid monohydrate and Na_2HPO_4 solutions and 0.005% (v/v) Tween 20 (Sigma-Aldrich, Poole, UK) in the correct proportions to ensure a final pH of: 3, 3.6, 4, 4.6, 5, 5.6, 6, 6.6, 7 and 7.6 (data not shown).

2.4. Detection of phosphorylation of intracellular signalling intermediates

2.4.1. Phosphokinase array

Upregulation of phosphorylation of intracellular kinases was detected using a Proteome Profiler Human Phospho-Kinase Array kit (Bio-Techne Ltd., Abingdon, UK), according to the manufacturer's instructions, and as previously described [17]. Briefly, HOMECs were seeded in 10 cm^2 petri dishes and cultured as above until confluent. Cells were starved overnight and then treated ± 50 ng/ml CathL, for 4 minutes and subsequently lysed. A BCA protein assay (Fisher Scientific, Loughborough, UK) was performed to quantify the total protein levels in each lysate. Controls received carrier alone. 200 µg of protein (lysate) was incubated with antibody coated membranes and levels of phosphorylated proteins were assessed by chemiluminescence detected on film. The relative expression of specific phosphorylated proteins was determined following quantification of spot density on scanned images by Image-J. The results are expressed as mean dot density (arbitrary units).

2.4.2. Cell based ELISA

Phosphorylation levels of ERK1/2 and AKT(S473) were measured using specific cell-based ELISA kits (Bio-Techne Ltd., Abingdon, UK) according to the manufacturer's instructions, and as previously described [17]. Cells were treated ± $VEGF_{165}$ (20 ng/ml, positive control) and CathL (50 ng/ml) in the presence or absence of MEK1/2 (upstream of ERK1/2) and AKT inhibitors at their given concentrations (Table 1) for 4 and/or 10 minutes. Multiple time points were tested for the phosphorylative status of kinases as activation of intracellular kinases can be transient [18]. The shorter time point (4 minutes) for CathL was selected because previous reports suggest that MAPK/ERK1/2 and AKT phosphorylation are maximum at 4-5 minutes [19]. Fluorescence intensity was measured and the results are expressed as fold change in phospho-ERK1/2 or -AKT relative to total ERK or AKT levels (compared to control).

2.5. HOMEC migration

Assessment of cellular migration was carried out using a Cultrex Cell 96 transwell migration assay (Bio-Techne Ltd., Abingdon, UK) as previously described [17]. Briefly, cells were incubated in growth factor-deprived media supplemented with 0.5% FCS overnight. Next, cells were seeded at a density of $5x10^4$ in the upper assay chamber and treated ± $VEGF_{165}$ (20ng/ml, positive control) and/or CathL (50ng/ml) and in the presence or absence of MEK1/2 and/or AKT(S473) inhibitors at their given concentrations (Table 1). Negative controls received carrier alone. After 6 hours incubation at 37°C, the bottom chambers were washed, followed by addition of cell dissociation solution/calcein AM for a further hour to label and detach migrated cells. Fluorescence in the bottom wells was read at Ex/Em: 485/520 nm.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (SD) and analysed using Mann-Whitney U test. A *p* value of less than 0.05 was considered statistically significant. For all data, *n* represents the number of wells or dishes tested under each condition and also the results from at least two primary cell populations.

3. Results

3.1. CathL induces proliferation of HOMECs

The tumour microenvironment is rich in tumour-secreted factors that activate normally relatively quiescent ECs, inducing angiogenesis which ensures a nutrient supply for the growing tumour. Cellular proliferation and migration are two key elements of this proangiogenic process. We have previously reported that CathL is secreted by EOC cells and since CathL has been reported to be involved in angiogenesis [20], initial studies examined the dose dependent (20, 50 and 80 ng/ml) proliferative effects of CathL on HOMECs. The concentration range was selected based on previously published concentrations of CathL concentration in the serum of ovarian cancer patients [14] and in the serum of patients with rheumatoid arthritis [21]. 50 ng/ml CathL induced a significant increase in HOMEC proliferation after 72 hours (147.0±25.8% vs control, [100%], p=<0.001; Fig. 1a), and thus this concentration was used for all further experiments.

CathL-induced (50 ng/ml) HOMEC proliferation was further confirmed using two separate methods: BrdU and CyQUANT, which demonstrated a significant increase in cellular proliferation. For instance, in response to CathL treatment, HOMEC proliferation significantly increased to 133.5±10.4% ($p<0.05$; Fig. 1b) and 109.1±5.0% ($p<0.01$; Fig. 1c) in BrdU and CyQUANT assays respectively, where all were normalised to control (100%).

3.2. CathL induces HOMEC proliferation via a non-proteolytic mechanism

Next, a series of experiments were performed to investigate whether mature CathL enhances HOMEC proliferation in a manner that is dependent on its proteolytic activity. Previously, CathL has been shown to be proteolytically active at neutral pH [22]. Thus, initial studies examined whether CathL was proteolytically active in the cell culture conditions studied. Preliminary studies confirmed that the pH of cell growth media in both the presence and absence of CathL remained at between pH 7.11 and 7.19 even over 72 hours of cell culture (Table 2). Using a fluorogenic substrate ZVA (5 µmol/l) CathL enzymatic activity was then tested over a range of pHs from 3 to 7.6 (data not shown) which included the published optimum pH for proteolytic activity (pH 5.5) and the pH neutral conditions confirmed above in cell culture (pH 7-7.6). CathL activity peaked at pH 5 and remained significant, though at a lower level, even at pH 7 (Fig. 2a), confirming previously published work suggesting CathL proteolytic activity at both optimum and neutral pHs [22]. When co-incubated with FY-CHO (10 µmol/l), a well-known inhibitor of CathL-proteolytic activity, CathL-mediated cleavage of the substrate was completely inhibited at pH 5 and 7 confirming the effectiveness of the inhibitor (Fig. 2a)

Fig. 2b demonstrates that CathL-induced proliferation of HOMECs was not inhibited by FY-CHO over a range of concentrations including 10 µmol/l which fully inhibited the enzymatic activity of CathL above (Fig. 2a). The data presented in Fig. 2c confirm that FY-CHO had no effect on HOMECs proliferation at any of the concentrations used i.e. between 0.1 µmol/l and 10 µmol/l.

These combined data indicate that CathL is not proteolytically active in the assay conditions studied (i.e. neutral pH in the presence of FY-CHO) and that therefore the proliferative effect of CathL in HOMECs is not likely to be the result of its proteolytic activity but rather via a non-proteolytic mitogenic mechanism.

3.3. CathL activates the proliferative kinases ERK1/2 and AKT (S473)

The observation that CathL exerts its mitogenic effects in HOMECs via a non-proteolytic mechanism raises the possibility that the protein acts as an extracellular ligand, interacting with an, as yet unknown, receptor to activate intracellular signalling pathways. This was initially investigated using a proteome-profiler phosphokinase array as a screening tool. Several kinases were identified to be phosphorylated in HOMECs during CathL treatment for 4 minutes. These included the known cell proliferative kinases ERK1/2 and AKT which demonstrated a 3-fold increase in phosphorylation, during CathL treatment compared to control (Fig. 3a).

To confirm this initial screen, a cell-based ELISA was carried out following 4 and 10 minutes incubation with CathL and $VEGF_{165}$, where $VEGF_{165}$ was the positive control. After 4 minutes treatment with CathL, there was a >2-fold and ~1.6-fold increase in ERK1/2 and AKT phosphorylation relative to the total ERK1/2 and AKT levels respectively and compared to control (untreated) (Fig. 3b, d). However, after 10 minutes incubation, phosphorylated levels of both ERK1/2 and AKT reduced to the basal level observed in untreated cells (Fig. 3c, e). Interestingly, although CathL-induced ERK1/2 phosphorylation was transient, $VEGF_{165}$-induced ERK1/2 phosphorylation was maintained for at least 10 minutes.

The validity of the cell-based ELISA kit was verified using known inhibitors of MEK1/2 and PI3K/AKT. Pre-incubation with non-toxic concentrations (determined during preliminary investigations, data not shown) of MEK1/2 inhibitors U0126 (10 μmol/l) and PD98059 (25 μmol/l) totally abolished the CathL (and $VEGF_{165}$) induced increase in phosphorylation (Fig. 4a, b). Similar results were observed in the AKT ELISA using LY294002, a PI3K inhibitor and MK2206, a selective AKT inhibitor (Fig. 4c, d). These data confirm that both drugs inhibit the PI3K/AKT pathway in HOMECs.

3.4. CathL-induced HOMEC proliferation is mediated via ERK1/2 pathway, and not AKT

The data presented above suggest that CathL-induced HOMEC proliferation involves the activation of ERK1/2 and AKT. This is confirmed by the data shown in Figure 5 showing that both MEK1/2 inhibitors, U0126 and PD98059, significantly reduced CathL-stimulated proliferation to levels equal to or below control levels. For example, in the presence of 10 µmol/l U0126, cell proliferation decreased to 88.2±11.4% (vs CathL 134.8±14.7%, $p<0.001$, Fig. 5a), all normalised to control. In the case of PD98059, cell proliferation was reduced to 64.4± 4.9% (Fig. 5b) at 25 µmol/l, compared to CathL alone (127.0±7.9%, $p<0.001$) (all expressed as percentage of control). A similar observation was made in HOMECs treated with CathL in the presence of the PI3K inhibitor LY294002 (102.0±5.9% vs 137.3±5.7% CathL-only treatment, $p<0.001$, both normalised to control, Fig. 5c). However, cell proliferation was not significantly altered in the presence of MK2206, a selective inhibitor of AKT (discussed later). For example, cell proliferation in the presence of CathL plus 5 µmol/l of MK2206 was 126.7±10.9% (Fig. 5d) compared with CathL-induced proliferation, 125.1±6.7% (all data normalised to control (100%)). Together, these data suggest that CathL induces HOMEC proliferation via a non-proteolytic mechanism that involves activation of intracellular pathways downstream of ERK1/2 phosphorylation and possibly PI3K.

3.5. HOMEC migration is induced by CathL treatment via the ERK1/2, but not the AKT pathway

Endothelial cell migration is another key step in tumour-angiogenesis. In an initial experiment, CathL significantly increased HOMEC migration by 197.4±49.6% ($p>0.001$; data not shown) compared to control (100%). This prompted an investigation into the downstream signalling cascades. Inhibitors of MEK1/2 completely abolished CathL-induced HOMEC migration to basal levels observed in control, untreated wells. For instance, in the presence of U0126 and PD98059, CathL-induced HOMEC migration reduced to 75.6±19.1% (Fig. 6a) and 91.5±14.1% (Fig. 6b) respectively, compared to CathL treatment alone (146.6±17.3%, $p<0.001$), all expressed as percentage of control (100%). In the case of PI3K/AKT inhibitors

LY294002 and MK2206, no significant reduction in CathL-induced HOMEC migration was observed. For instance, in the presence of LY294002 and MK2206, CathL-induced cell migration was 188.8±46.5% (Fig. 6c) and 175.3±27.0% (Fig. 6d.) respectively, compared to CathL treatment (199.2±62.2%, $p<0.001$), all expressed as percentage of control (100%). These data combined with the ELISA data (Fig. 4), suggest that CathL induces HOMEC migration via a pathway that requires activation of the ERK1/2 but not the AKT(S473) pathway.

4. Discussion

The high mortality rate of ovarian cancer sufferers can be explained by late diagnosis at an advanced disease state, with widespread metastasis within the peritoneal cavity. This is primarily due to vague symptoms at an early stage of the disease, and hence diagnosis can be clinically challenging. Anti-angiogenic therapies that target the newly forming vasculature of the growing secondary foci have primarily targeted the VEGF/VEGFR2 axis and have had disappointing outcomes. This may be due to the compensatory contribution of other pro-angiogenic factors. Indeed, we have previously reported that omental angiogenesis in EOC metastasis could occur independently of $VEGF_{165}$ signalling, potentially through alternative pro-angiogenic factors including CathL. *In vitro* CathL is secreted from ovarian tumour cells and *in vivo* the overexpression of CathL is observed in omental tissue of patients with serous ovarian carcinoma [9, 15]. Additionally, CathL induced pro-angiogenic phenotypic changes in HOMECs [9], although the full effect of CathL and the mechanisms by which it acts to induce these cellular changes remain unknown. In this study, we demonstrate for the first time that CathL induces significant proliferation and migration via a mechanism that is not dependent on the proteolytic activity of the enzyme. Further to this, we also demonstrate that CathL induction of proliferation and migration was mediated via activation of the ERK1/2 pathway. These data support the hypothesis that CathL secreted from EOC metastasising to the omentum contributes to angiogenesis in the growing secondary tumour foci within the omentum.

CathL is a lysosomal ubiquitous cysteine proteinase that plays an important role in degrading endocytosed proteins as well as intracellular proteins [11, 12]. CathL is

translated as preprocathepsin L (ppCathL) and processed into procathepsin L (pCathL) in the rough endoplasmic reticulum with a molecular mass of 30kDa and a two-chain form with molecular masses 25kDa and 5kDa [23, 24]. It is then transported to endosome/lysosomes via the mannose-6-phosphate/receptor (M6P/M6PR) pathway [25]. CathL contains covalently N-linked oligosaccharides including a mannose moiety that is phosphorylated by phosphodiesterases in the *cis* Golgi [26]. These M6P groups are recognised by an M6PR protein in the *trans* Golgi network (TGN), and facilitate the delivery of the protein to lysosomes (via endosomes) [27]. The cathepsins dissociate from the receptors at low lysosomal pH, and the phosphate group is removed from the M6P moiety by a lysosomal acid phosphatase [28]. However, CathL has also been shown to be secreted out of the cell. CathL was first identified as a major secreted protein from a transformed murine fibroblast cell line [29]. However, the mechanism of secretion of CathL remains a mystery. It has been shown that CathL has only one M6P residue, and hence it's lower affinity for M6PR, as opposed to enzymes with two M6P residues such as cathepsin D [30-32].

A role for extracellular CathL has been linked to tumour invasion and metastasis, particularly by degrading several ECM components such as proteoglycan, aggrecan, elastin, laminin, fibronectin and collagens: I, II, IX, XI [33-38]. Additionally, CathL has been shown to have a role in cell proliferation. A role for extracellular CathL in metastasis is less well studied, but our observation that CathL is secreted from EOC cancer cells led us to investigate its potential role in inducing a pro-angiogenic phenotype i.e. proliferation and migration, in disease-relevant HOMECS. We showed that CathL significantly stimulated proliferation in HOMECs within the reported physiological concentration range. We next examined whether CathL acts via a proteolytic- or non-proteolytic mechanism to induce this proliferation.

Initial studies using ZVA, a CathL-specific fluorogenic substrate showed that CathL was proteolytically active across the pH range starting from its optimum pH (4.5) up to pH 7.6; which included the pH of cell culture media throughout a typical proliferation experiment (demonstrated to be between 7.11 and 7.19). These data are supported by previous findings [39], demonstrating CathL proteolytic activity at pHs between 7 and pH 8 and suggested that CathL is catalytically active in our cell culture conditions.

We then examined whether FY-CHO, a selective, potent and non-cell permeable inhibitor of CathL catalytic activity, could inhibit CathL-induced proliferation in HOMECS. FY-CHO has previously been shown to be highly active against extracellular CathL. For instance, it has been extensively used to inhibit CathL mediated *in vitro* migration and invasion of breast and prostate cancer cells [40]. Here, this inhibitor was primarily chosen over cystatin C, a commonly used CathL inhibitor, because cystatin C elicits non-selective inhibition on all cysteine proteases.

Preliminary studies, using ZVA, confirmed that FY-CHO completely inhibited CathL proteolytic activity at pH 7, however the same concentration (10 µmol/l), of FY-CHO had no effect on CathL-induced HOMEC proliferation, suggesting that CathL induces proliferation in these cells via a novel proteolytic-independent mechanism i.e. acts as an extracellular ligand.

This led us to investigate the possible intracellular pathways activated by CathL in HOMECs. To address this, we investigated activation of possible downstream proliferative kinases in these cells using a human proteome profiler that identifies the phosphorylation status of 43 intracellular kinases. Both ERK1/2 and AKT phosphorylation levels were upregulated in HOMECs following CathL treatment, compared to control and these array data were confirmed using live cell-based ELISAs. Involvement of these kinases in mediating cellular proliferation was confirmed using well-known MEK1/2 (upstream of ERK1/2) inhibitors (U0126 and PD98059) and PI3K/AKT kinase inhibitors (LY294002 and MK2206). Initial ELISA experiments confirmed that these inhibitors fully abolished CathL-induced cellular ERK1/2 and AKT phosphorylation. In the subsequent experiments, we showed that the two MEK1/2 inhibitors significantly reduced or abolished CathL-induced HOMEC proliferation over 72 h. This observation was replicated in in the presence of LY294002 (PI3K inhibitor) but not with the selective AKT inhibitor MK2206. This is perhaps not surprising as LY294002 is known to cross-react with the ERK1/2 pathway where it inhibits ERK1/2 phosphorylation [41]. Thus, it is possible that LY294002 in fact inhibited activation of ERK1/2 and reduced HOMEC proliferation, and that AKT is not involved in the induction of cell proliferation as the AKT-specific inhibitor failed to reduce this proangiogenic response. Taken together, these data suggest that the ERK1/2 pathway is involved in the induction of HOMEC proliferation by exogenous CathL.

The observation that CathL induces intracellular activation of the MAPK ERK1/2 and PI3K/AKT pathways suggests the involvement of a cell surface receptor that initiates intracellular signalling following interaction with CathL. To date no CathL-activated receptors have been identified in ECs. However, ECs do express a number of receptors that are known to be upstream of both signalling pathways e.g. VEGFR2 and the epidermal growth factor receptor, and investigating possible receptor targets is a current focus of our research.

We also showed that CathL induced migration in HOMECs, a second key pro-angiogenic endothelial response. Investigation of the signalling pathways involved in the migratory response showed that CathL-induced HOMEC migration was only mediated via activation of the ERK1/2, but not the PI3K/AKT pathway. This is, to our knowledge, is the first report to observe such phenomenon.

Taken together our data suggest that CathL may be an important pro-angiogenic factor in omental metastasis of EOC. It is secreted from EOC and induces pro-angiogenic phenotypic changes in the local omental microvasculature. These observations are consistent with reports showing an increased level of secreted CathL in the sera of malignant epithelial ovarian cancer patients [14, 42], which correlated with a significant increase in the expression of CathL mRNA levels in tumours. Both serum levels and mRNA expression were higher in patients with malignant EOC than those with benign tumours or normal ovarian tissue. CathL has also been shown to be involved in the invasion and metastasis of EOC, and hence has been suggested to be a marker of advanced staged ovarian cancer [14]. This was supported by our published work demonstrating that the endothelium of vessels within omentum hosting metastatic ovarian high-grade serous carcinoma expressed significantly increased CathL *in vivo* compared with omentum from control patients with benign ovarian cystadenoma [15]. In other cancer types over-expression of CathL was linked to metastasis following ras transformation of NIH/3T3 cells *in vitro* [43] and non-metastatic melanoma cells were converted to a metastatic state when over-expressing CathL *in vitro* [44]. It was also shown that CathL is involved in B16F10 melanoma cell invasion (*in vitro*), particularly through cell migratory influences [45]. It is known that CathL is secreted in different forms into the extracellular space in both physiological and pathological conditions and

retains its function as a protease which degrades ECM, which may allow cancer cells to invade surrounding tissue.

A role for CathL in angiogenesis is a relatively new observation and, interestingly, evidence from different models suggests that CathL may be both pro-and anti-angiogenic. For instance, recently, CathL derived from skeletal muscle cells transfected with bFGF was shown to promote migration of human umbilical vein ECs (HUVECs) *in vitro* [46]. Cell migration, a key component of angiogenesis, was tested in the presence of a cell impermeable CathL-proteolytic inhibitor FY-CHO and CathL for 12 hours. The data revealed a significant reduction in HUVEC migration, suggesting that CathL influences cell migration via its proteolytic-dependent mechanism. Subsequently, CathL was found to activate c-Jun N-terminal kinase (JNK) in migratory HUVECs. However, the exact role of CathL in activating the JNK pathway has not been elucidated [46]. Intriguingly, endothelial progenitor cells (EPCs) have been reported to produce CathL which in turn induced angiogenesis. Urbich *et al.* showed that EPCs were able to stimulate neovascularisation and blood flow in the ischaemic murine hind leg after injection into the affected leg [47]. Mature CathL was shown to remain proteolytically active extracellularly at neutral pH by the chaperone action of a p41 splice variant of the MHC class II-associated invariant chain [22], which indeed is strongly expressed in EPCs [47]. Such activity may facilitate EPC invasion and neovascularisation. Furthermore, mice treated with CathL-deficient bone marrow cells demonstrated a significant reduction in angiogenesis [47]. Another study also showed that CathL expressed in EPCs cells plays a critical role in intraocular angiogenesis [48]. In contrast, CathL was also shown to be antiangiogenic. For instance, both secreted and intracellular CathL have been shown to release endostatin, a potent inhibitor of angiogenesis, by cleaving ECM collagen [49]. Since the tumour microenvironment provides a slightly acidic milieu, CathL can efficiently cleave collagen even outside the cells. However, in other studies, CathL had no effect on angiogenesis. For example, Gocheva *et al.* demonstrated that CathL had no significant effects in altering microvascular density in pancreatic cancer in mice [50].

Conclusion

Taken together, the data presented here suggest that ovarian tumour-secreted CathL is a pro-angiogenic factor that induces proliferation and migration in HOMECs via a

non-proteolytic mechanism. These novel findings may contribute to the clinicopathology of advanced stage ovarian carcinoma where metastatic ovarian cancer causes extensive vascularisation of omental lesions, increasing the ability of the secondary tumour to survive and spread to other organs. As transcoelomic metastasis requires tumour angiogenesis, CathL, secreted from ovarian cancer cells, in cooperation with other cells present in the omentum may facilitate cellular angiogenesis in HOMECs. This may highlight CathL and its downstream pathways as novel anti-tumourigenic/anti-angiogenic therapeutic targets in the treatment of ovarian cancer.

Abbreviations

EOC, epithelial ovarian cancer; CathL, cathepsin L; EC, endothelial cell; ERK1/2, extracellular signal regulated kinase; AKT, protein kinase B; ECM, extracellular matrix.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MZIP performed experiments, collected results, did the data analysis and drafted the manuscript, NJG, MH and JLW supervised all the work and revised the manuscript.

Consent for publication

All the authors have read and approved the manuscript.

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Table 1. Concentrations of treatments added to cell proliferation assay.

Treatments	**Purpose**	**Concentration(s)**	**Source**
Recombinant $VEGF_{165}$	Positive control	20 ng/ml	Peprotech (London, UK)
Recombinant CathL from human liver	Treatment	20, 50, 80 ng/ml	Sigma-Aldrich (Poole, UK)
FY-CHO	CathL inhibitor	0.1, 1 and 10 µmol/l	Santa Cruz Biotechnology, Inc. (Heidelberg, Germany)
U0126	MEK1//2 inhibitor	10 µmol/l	Stratech (Suffolk, UK)
PD98059	MEK1/2 inhibitor	25 µmol/l	Stratech (Suffolk, UK)
LY294002	PI3K inhibitor	25 µmol/l	Stratech (Suffolk, UK)
MK2206	AKT inhibitor	5 µmol/l	Stratech (Suffolk, UK)

Table 2: **pH of cell culture media and supernatant during CathL treatment.** Cells were seeded in 6 well plates and treated with or without CathL (50 ng/ml) for 24, 48 and 72 hours. Media were collected and their pH was measured. n.d. denotes not determined.

	pH			
	0h	**24h**	**48h**	**72h**
Basal mv2	7.34	n.d.	n.d.	n.d.
Untreated	n.d.	7.19	7.13	7.12
CathL	n.d.	7.19	7.13	7.11

Fig. 1. **CathL increases proliferation of HOMECs.** Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000 cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without various concentrations of CathL and incubated for a) 72 hours. WST-1 kit was used to assess cellular proliferation based on absorbance using a PHERAstar BMG plate-reader at 450 nm (n = 15-20). b) Cell proliferation was tested using 50 ng/ml of CathL (BrdU). A commercially available BrdU reagent was added to the wells for the last 24 hour incubation and cellular proliferation was assessed (according to the manufacturer's instructions) at 48 hours after treatment based on fluorescence intensity using a SpectraMax plate-reader at Ex/Em of 450/550 nm (n = 6). c) HOMEC proliferation was examined at 50 ng/ml of CathL (CyQUANT). A commercially available CyQUANT reagent was used to assess cell proliferation after 72 hour treatment based on fluorescence intensity using FLUOstar BMG plate-reader at Ex/Em: 485/530 nm (n = 20). Results are mean ± SD and shown as percentage of the control, *p<0.05, **p<0.01 and ***p<0.001 vs control (100%). n.s. denotes not significant.

Fig. 2: **FY-CHO inhibits CathL proteolytic activity at pH 7, but does not inhibit CathL-induced HOMEC proliferation.** a) CathL proteolytic activity is inhibited at both pHs 5 and 7. A specific fluorogenic substrate Z-Val-Val-Arg-AMC (Z-VVR-AMC, ZVA, 5 µmol/l) was incubated ± CathL (50 ng/ml) and in the absence or presence of FY-CHO (10 µmol/l) at both pH 4 and 7. Fluorescence signals were measured immediately using a SpectraMax plate reader at Ex/Em: 365/440. Control wells contained pH buffer and substrate and/or inhibitor. The data are represented as percentage of control. *p<0.05, ***p<0.001 vs control (substrate) (100%); #p<0.05, ###p<0.001 vs CathL + substrate (expressed as % of control), n = 3. b + c) FY-CHO does not inhibit CathL induced proliferation. Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000 cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without CathL (50 ng/ml) and incubated for 72 hours ± increasing concentrations of FY-CHO as indicated- b). c) Cells were seeded as for (b) and treated with FY-CHO alone for 72 hours. WST-1 assay was used to assess cellular proliferation based on absorbance using a PHERAstar BMG plate-reader at 450 nm. Control wells contained 0.1% DMSO (carrier only). Results are mean ± SD and shown as percentage of the control, ***p<0.001 vs control (100%); n = 8-16. n.s. denotes not significant.

Fig. 3. **CathL induces activation of the intracellular kinases ERK1/2 and AKT in HOMECs.** a) CathL induces phosphorylation of ERK1/2 and AKT(S473) in HOMECs as assessed by proteome profile screening. Phosphorylation status of 43 intracellular kinases was assessed in lysates from cells treated ± CathL for 4 minutes. The results of 1 minute exposure are expressed as mean dot density (arbitrary units). The relative expression of specific phosphorylated proteins was determined following quantification of scanned images. b-e) CathL induces phosphorylation of ERK1/2 and AKT in HOMECs as assessed by live-cell ELISAs. Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000 cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without 50 ng/ml of CathL or 20 ng/ml of VEGF (positive control) and incubated for 4 or 10

minutes. ERK1/2 (b, c) and AKT (d, e) phosphorylation was examined after 4 minutes (b, d) and 10 minutes (c, e) treatments. Commercially available cell-based ELISAs were used for the determination ERK1/2 and AKT(S473) phosphorylation level. The ELISA experiments were carried out on two cell batches. The data is represented by fold change in phosho-ERK1/2/AKT relative to total ERK1/2/AKT (compared to control). Results are mean ± SD, **p<0.01 vs control (dotted lines); n = 4-6. n.s. denotes not significant.

Fig. 4. **ERK1/2 and AKT phosphorylation was not inhibited by their corresponding inhibitors in intact HOMECs.** After overnight starvation in media supplemented with 2% FCS, cells were pre-incubated with the MEK1/2 inhibitors a) U0126 (10 µmol/l) and b) PD98059 (25 µmol/l) or PI3K/AKT inhibitors c) LY294002 (25 µmol/l) and d) MK2206 (5 µmol/l) for (a + b) 20-30 minutes or (c + d) 2.5 hours, and then co-treated ± 50 ng/ml of CathL or 20ng/ml of VEGF for 4 minutes. Commercially available cell-based ELISAs were used for determination of ERK1/2 (a and b) and AKT (c and d) phosphorylation levels. The data in all are represented as fold change in phosho-protein relative to total protein (compared to control). Results are mean ± SD, *p<0.05, **p<0.01 vs control (1-fold), #p<0.05 vs VEGF/CathL (normalised to control), n = 4. The dotted lines represent basal level (control) of phosphorylation status in untreated HOMECs.

Fig. 5. **CathL-induced HOMEC proliferation is mediated via activation of the ERK1/2 and PI3K pathways, but not AKT pathway.** After overnight starvation in media supplemented with 2% FCS, cells were treated with or without CathL (50 ng/ml) ± a) U0126 (10 µmol/l), b) PD98059 (25 µmol/l), c) LY294002 (25 µmol/l) and d) MK2206 (5 µmol/l) and incubated for 72 hours. WST-1 assay was used to assess cellular proliferation. Results are mean ± SD and shown as percentage of the control, n.s., **p<0.01, ***p<0.001 vs control (100%), ###p<0.001 vs CathL (normalised to control 100%), n = 7-15. n.s. denotes not significant.

Fig. 6. **CathL induces HOMEC migration via activation of the ERK1/2 (a, b), but not the AKT pathway (b, c).** Pre-treated (with corresponding kinase inhibitor) HOMECs were seeded in the upper transwell chamber and treated with or without CathL (50 ng/ml) ± a) U0126 (10 µmol/l) and b) PD98059 (25 µmol/l) or c) PI3K and d) AKT inhibitors LY294002 (25 µmol/l) and MK2206 (5 µmol/l), respectively in media containing 0.5% FCS. The lower well contained correspondent treatments. After 6 hours, migrated cells were stained with calcein AM and fluorescence was quantified using a FLUOstar plate reader at Ex/Em: 485/520. Results are mean ± SD and shown as percentage of the control, *p<0.05, **p<0.01, ***p<0.001 vs control (100%), ###p<0.001 vs CathL (normalised to control (100%)), n = 6-12. n.s. denotes not significant.